# Recent advances in identification of male specificity determinant and its function in S-RNase-mediated gametophytic self-incompatibility

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**Abstract:** S-RNase-mediated gametophytic self-incompatibility (GSI) is controlled by a multiallelic S-locus at which two separate genes, the female (pistil) and male (pollen) specificity determinants, are tightly linked. This review described both the identification of pollen specific F-box genes, *SLF/SFBs*, in Antirrhinum, Petunia and *Prunus* species and the demonstration of *SLF/SFB* as pollen determinant together with their functions in GSI response. Recent studies of how the pollen determinant functions in pollination reaction revealed that pollen determinant interacted with S-RNases in a non-allele-specific manner. It targeted all of the non-self S-RNases for ubiquitination through a functional SCF complex and subsequent degradation via 26S proteasome pathway in compatible reaction. It allows pollen tube to reach into the embryo sac and to finish double fertilization. In incompatible response, the intact self S-RNases were left to function as a cytotoxin that degrades self-pollen tube RNA, resulting in the cessation of pollen tube growth.

**Keywords:** Gametophytic self-incompatibility; Pollen specific F-box genes; Male determinant; SCF complex; 26S proteasome pathway

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## Introduction

Self-incompatibility (SI) is a mechanism in flowering plants that prevents self-fertilization and promotes out-crossing (de Nettancourt 2001), thereby avoiding species retrogression in evolution of plants. To date, three plant families, Solanaceae (Anderson *et al*. 1986; McClure 1989), Scrophulariaceae (Xue *et al*. 1996) and Rosaceae (Sassa *et al*. 1992), possess a type of SI, termed gametophytic self-incompatibility (GSI). GSI behavior of pollen tube is determined by its own S genotype, rather than by the S genotype of the pollen-producing parent (Roalson and McCubbin 2003). That is, when an S gene of pollen matches one of S genes of the pistil, the pollen is recognized as "self" and rejected by the pistil. GSI is controlled by an apparently single multiallelic locus, designated S-locus (de Nettancourt 1977), which determines the specificity of the response. Recent studies on pistil- or pollen-specific self-compatible (SC) mutants (Thompson *et al*. 1991; Sassa 1997; Golz *et al*. 1999) and transformation experiments with solanaceous species (Lee *et al*. 1994; Murfett *et al*. 1994; Dodds *et al*. 1999) have demonstrated that two separate genes encoding male (pollen) and female (pistil) determinants at the S-locus control male and female specificities of SI response, respectively. Thus, the term "haplotype" is adopted to describe the variant of the S-locus, and the term "alleles" are used to describe variants of an S-locus gene. It appears that a direct interaction between the male S gene product and female determinant is required for the SI reaction (McCubbin and Kao 2000). Thus far, the female specificity determinants have been identified as a class of polymorphic proteins that have ribonuclease activity, thus they were labeled S-RNases (McClure 1989). In recent years, a series of F-box motif-containing genes, designated as *SLF/SFBs* (S-locus F-box), have been discovered while investigating GSI of plants and searching for male S determinants in Antirrhinum, Petunia and *Prunus* species. In addition, biochemical and molecular biological experiments have been performed to confirm that the expression products of *SLF/SFBs* in pollination reaction carry the specificity for substrate recognition, and directly participate in the protein-S-RNase interaction likely by forming a SCF complex, causing non-self S-RNases degradation via ubiquitin-mediated 26S proteasome pathway.

## Identification of pollen specific F-box genes

The cloning of female S-RNase genes initially depended on S glycoprotein separation and amino acid sequencing, whereafter the probe was designed to obtain pistil specific cDNA. Anderson *et al*. (1986) ultimately obtained the first cDNA clone encoding S-RNase, namely female S determinant, from the style of *Nicotiana alata*. Similar approach was used to identify the pollen S proteins and to isolate their cDNAs from pollen. Nevertheless, it quickly became obvious that the pollen S protein is not as abundant as S-RNases, thus different approaches would be needed.

As predicted before, male S gene must be linked to the S-RNase gene, highly polymorphic, and specifically expressed in pollen (Thompson and Kirch 1992; Kao and McCubbin 1996), based on which Lai *et al*. (2002) attempted to search for pollen S genes by investigating genomic structure of the S-locus in Antirrhinum. The authors determined the 64 kb sequences of BAC clones that contain the flanking regions of the $S_2$-RNase gene. As a result, a novel gene with the full length of 1 986 bp, designated *AhSLF* (*A. hispanicum SLF*), encoding an F-box-containing protein, was identified. *AhSLF-*$S_2$ encodes a polypeptide of 376 amino acids with a conserved F-box domain in its amino-terminal part. It is located 9 kb downstream of *$S_2$-RNase* gene and specifically expressed in tapetum, microspores and pollen grains in an allele specific manner.

In *Petunia inflata*, a member of Solanaceae, through a thorough search of the pollen S determinant in a huge S-locus region,

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328 kb BAC contig of $S_2$-haplotype, Sijacic *et al*. (2004) identified a polymorphic F-box gene, named *$P_i$SLF*, around 161 kb downstream of the *S-RNase* gene. Although the genomic region outside this contig contained two more polymorphic F-box genes that were genetically linked to the S-locus, the *$P_i$SLF* exhibited the highest sequence diversity (10.3%–10.6%). Through RNA gel blot, the expression of *$P_i$SLF* was detected only in anthers, mature pollen, and pollen tubes.

Hereafter, pollen-expressed F-box genes *SLF/SFBs* in *Prunus* species were reported one after another. Ushijima *et al*. (2003) sequenced completely a 70 kb segment of the S haplotype-specific region containing the *S-RNase* gene of the Rosaceous species almond (*Prunus dulics*), and two pollen-expressed F-box genes were found. One of them, named *SFB*, was expressed specifically in pollen and the deduced amino acid sequences of which display 23.6% to 31.6% allelic sequence diversity, which is higher than that of *SLF* (4.9%), the other F-box gene. The authors further confirmed that *SLF* is expressed also in pistil, but *SFB* specifically is expressed in pollen, and located within –30 kb of the *S-RNase* gene, suggesting that it is inherited with *S-RNase* gene as a unit. *SFB* is considered as a candidate gene of male determinant. The F-box motif is conserved at the N-terminal regions of all SFBs, and two regions at the C terminus are quite variable. By the same methods, Entani *et al*. (2003) investigated the genomic structure of the S-locus region of Japanese apricot (*Prunus mume*), and sequenced the 64 kb segment of cosmid clone covering *$S_1$*- and *$S_7$*-*RNase* genes. As a result, 4 F-box genes around the *S-RNase* gene were identified. Among them, only one F-box gene, termed *SLF* (S-locus F-box), fulfilled the conditions for a pollen S-determinant gene: it is located within the highly divergent genomic region of the S-locus together with the *S-RNase* gene, exhibits S haplotype specific diversity (78%–81% amino acid identity) among three analyzed S haplotypes, and is specifically expressed in pollen. Thus, *SLF* is viewed as a prime candidate for the pollen S determinant gene of SI. Around the same time, during an attempt to compare the $S_6$-haplotypes of an SI sweet cherry (*Prunus avium*) cultivar and an SC sour cherry (*Prunus cerasus*) cultivar, Yamane *et al*. (2003a) found a novel gene for a protein with an F-box motif, *Pa-SFB$^6$*, that is located about 380 bp downstream of *S$^6$-RNase*, shows 69.3%–78.0% homology with *Pd-SFBs* of almond at the amino acid level, and is specifically expressed in pollen grains through RT-PCR assay.

The final pollen specific F-box gene was reported by Romero *et al*. (2004) via sequencing 21 kb in total of the S-locus region in 3 different apricot (*Prunus armeniaca L.*) S haplotypes. These fragments contain genes homologous to the *S-RNase* and F-box genes found in other *Prunus* species. The physical distance between the F-box and the *S-RNase* genes was determined exactly in the $S_2$-haplotype (2.9 kb), confirming that these genes are linked. The apricot F-box allelic variants (*$SFB_1$*, *$SFB_2$* and *$SFB_4$*) are also highly haplotype-specific (79.4% amino acid identity). Organ specific-expression analysis reveals that *$SFB_1$* and *$SFB_2$* are expressed merely in pollen, but not in styles or leaves. These results support these genes as candidates for the pollen S-determinants of GSI in apricot.

## Demonstration of *SLF/SFB* as male specificity determinant in GSI

Sequence analysis and expression assay of *SLF/SFBs* from *Prunus* species suggest that they fulfill all conditions of the pollen *S* determinant as described above, nevertheless they are only determined as the candidates for pollen S genes attributed to a lack of further work on their functions. The data presented by Yamane *et al*. (2003b) were consistent with this notion. They investigated S-locus region of mutated S$^6$-haplotype, namely S$^{6m}$-haplotype, of SC sour cherry. Inverse PCR for the flanking regions of *S$^6$-RNase* in the S$^6$-and S$^{6m}$-haplotypes revealed an approximately 2 600 bp insertion present at approximately 800 bp upstream of the *S$^6$-RNase* in the S$^{6m}$-haplotype, which is a possible cause of suppression of the transcription of S$^6$-RNase. *SFB$^6$* was presented downstream of *S$^6$-RNase* in both the S$^6$-and S$^{6m}$-haplotypes and expressed in the same way, supporting the idea that *SFB* is a good candidate for pollen S gene in *Prunus*.

In addition, Ushijima *et al*. (2004) investigated two SC haplotypes, S$^{4'}$ and S$^f$, from sweet cherry and Japanese apricot, respectively, which are considered to be two SC mutants. They detected that a 4 bp deletion upstream from the HVa coding region of *SFB$^{4'}$* causes a frame-shift that produces transcripts of a defective *SFB* lacking the two hypervariable regions, HVa and HVb. Similarly, the presence of a 6.8 kbp insertion in the middle of the *SFB$^f$* coding region leads to transcripts for a defective SFB lacking the C-terminal half that contains HVa and HVb. The loss of pollen function of sweet cherry and Japanese apricot in GSI reaction implicates that *SFB/SLF* likely encoded male S determinant.

The conclusive evidence that *SLF/SFB* encodes the pollen S determinant was ultimately obtained from transformation experiments in *Petunia inflata* (Sijacic *et al*. 2004). To ascertain whether *$P_i$SLF* encodes the pollen S determinant, a well-documented phenomenon termed "competitive interaction" was utilized. Competitive interaction is often observed in tetraploid plants. Among the diploid pollen grains produced, those carrying two different S haplotypes (heteroallelic pollen), but not two of the same S haplotypes (homoallelic pollen), fail to function in SI (de Nettancourt 2001; Entani *et al*. 1999). In accordance with this phenomenon, the transformation of $S_1S_1$, $S_1S_2$, and $S_2S_3$ plants with the $S_2$-allele of *$P_i$SLF* (*$P_i$SLF$_2$*) caused breakdown of their pollen function in SI. Furthermore, genotypic analyses of the progeny from self pollinations of $S_1S_2$/*$P_i$SLF$_2$* and $S_2S_3$/*$P_i$SLF$_2$* revealed that only $S_1$ and $S_3$ pollen carrying the *$P_i$SLF$_2$* transgene (corresponding to heteroallelic pollen), but not $S_2$ pollen carrying *$P_i$SLF$_2$* (corresponding to homoallelic pollen), became SC. These results definitely demonstrate that *SLF/SFB* is the long-sought pollen S-determinant.

Transformation experiment of polymorphic F-box (SLF) protein AhSLF-$S_2$ of *Antirrhinum hispanicum* was also performed by Qiao *et al*. (2004a), and they transformed an Antirrhinum transformation-competent artificial chromosome (TAC) clone that contains both *AhSLF-$S_2$* and *Ah$S_2$-RNase* into a SI line of *Petunia hybrida*. In a separate experiment, they transformed the *AhSLF-$S_2$* cDNA driven by a tomato (*Lycopersicon esculentum*) pollen-specific promoter *LAT52* into the same line. In both cases, the introduced genes were correctly expressed in the reproductive tissues of transgenic Petunia plants that significantly all became SC due to the loss of pollen function of SI according to pollination tests. Importantly, expression of *AhSLF-$S_2$* does not interfere with the expression of endogenous *SLF* or *SLF*-like genes in pollen. These results presented by Qiao *et al*. are consistent with competitive interaction as described above which causes breakdown of pollen function in SI, confirming that pollen specific F-box gene *AhSLF-$S_2$* is indeed pollen S determinant,

and controls the pollen function of S-RNase-mediated GSI.

Taken together, sequence analysis of *SLF/SFB* genes, structural investigation of S haplotye of mutants in *Prunus* species along with recent studies in Petunia and Antirrhinum strongly pointed to polymorphic *SLF/SFB* as the pollen S determinant.

## Male S determinant likely mediates non-self S-RNases degradation through ubiquitin/26S proteasome pathway

Recent studies revealed that the F-box-containing protein is involved in the ubiquitin/26S proteasome pathway of protein degradation (Deshaies 1999). This degradation system has been shown to play a key role in many cellular processes in plants including flower development and signal transduction (Xiao and Jang 2000). It uses E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) to catalyze the formation of polyubiquitin chains on specific substrates for degradation by the 26S proteasome (Fig. 1) (Bai *et al*. 1996). An F-box protein is a component of one class of conservative E3, called SCF, which also consists of Skp1, cullin (Cul1), and a RING-HC finger protein (Rbx1) (Tyers and Jorgensen 2000). In this pathway, the F-box motif interacts with Skp1, and a separate region recognizes and interacts with specific target substrate that is to be ubiquitinated at the C terminus. Poly ubiquitinated proteins are subsequently recognized and degraded by the 26S proteasome, and ubiquitins were released (Fig. 2).

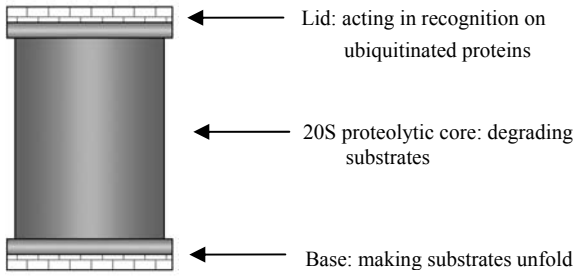


**Fig.1 Structure and function of 26S proteasome**

**Note**: 26S proteasome comprises two parts. One is 20S proteolytic core that functions as substrate destruction. The other is 19S regulatory particle containing two sub-complexes, "lid" and "base". "Lid" consisting of 18 subunits is in charge of recognition for ubiquitinated proteins, thus it carries the specificity of 26S proteasome. "Base" comprises 6 subunits and the function of which is to make substrate unfold, catalyzing the destruction of substrate.

Identification of pollen S determinant as an F-box protein reveals that polymorphic pollen specific *SFB/SLF* appears to act as a component of SCF complex, which indicates ubiquitin-mediated 26S proteasome pathway is likely involved in some significant proteins degradation, thus causing recognition response after pollination in S-RNase-mediated GSI.

Sims and Ordanic (2001) first proposed that ubiquitination and protein degradation may play a role in the SI reaction in *P. hybrida*. Using yeast two-hybrid technique they identified a pollen expressed protein (PhSBP1) with a RING-finger domain that binds to S-RNases in a non-allele-specific manner. Because many proteins with a RING-finger domain participate in E3 ubiquitin ligase complexes, like F-box proteins, they suggest that PhSBP1 is a candidate for the general inhibitor of S-RNases, participating in protein degradation in SI.

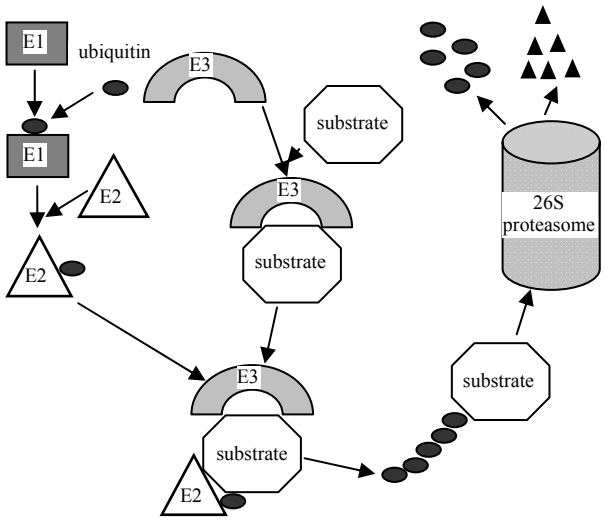


**Fig.2 Ubiquitin-mediated protein degradation pathway**

The data presented by Qiao *et al*. (2004b) provide additional evidence that pollen specific SLF protein is associated with 26S proteasome degradation. Coimmunoprecipitation and yeast two-hybrid assays were performed by the authors to demonstrate that AhSLF-$S_2$ physically interacts with S-RNases as well as CULLIN1 (CUL1)-like and ASK1-like proteins, together possibly forming an SCF complex in Antirrhinum. They further showed that compatible (non-self) pollination was blocked after treatment with proteasomal inhibitors, while the incompatible pollination reaction was unaffected, suggesting that 26S proteasome activity is required for compatible pollination. Finally, a series of experiments showed that the ubiquitination level of style proteins was higher, and S-RNases appear to be subsequently reduced after compatible pollination relative to incompatible pollination reactions.

Furthermore, two variable regions were observed at the C terminus of SLF/SFB in *Prunus* species. This observation implicates (Ushijima *et al*. 2003) that the regions are responsible for the discrimination between self- and non-self S-RNases, which agrees with the results shown by Qiao *et al*. According to studies of Qiao *et al*., it could be concluded that during the process of pollen tube growth in style after pollination, both types of S-RNases, self and non-self S-RNases, enter pollen tube (McFadden *et al.* 1992; Luu *et al*. 2000), and pollen S determinant likely function as a component of SCF complex and interacts with S-RNases including self and non-self S-RNase, whereas it more likely targets non-self S-RNases for selective ubiquitination and destruction by 26S proteasome, thus allowing pollen tube to reach into the embryo sac and to finish double fertilization. Self S-RNase activity was free of ubiquitination to function as a cytotoxin that degraded self-pollen tube RNA, arresting the growth of self-pollen tube (Fig. 3).

## Discussion

Since the discovery of the first pollen-expressed F-box gene in Antirrhinum, *Ah-SLF*$_2$, other pollen specific F-box genes were identified by researchers via sequencing the S-loci of *Prunus*

species, like *Prunus dulics*, *Prunus avium*, *Prunus cerasus*, *Prunus mume* and *Prunus armeniaca*. Based on sequence analyses of S-locus in conjunction with studies in mutants of cherry and Japanese apricot, Yamane *et al.* (2003b) and Ushijima *et al*. (2004) proposed that pollen *SFB/SLF* is the prime candidate of pollen S determinant. Hereafter, transformation experiments in *Petunia inflata* and Antirrhinum definitely confirm that *SLF/SFB* encodes the pollen S determinant. Identification of pollen S determinant enables us to be closer to understanding of S-RNase-mediated SI than ever. It is unclear, however, that how the pollen S determinant functions in SI and how SI reaction is manifested at the molecular level. Current studies suggest that pollen S gene product functions as a E3 by forming a SCF complex together with other subunits and participates in ubiquitin-mediated 26S proteasome pathway that targets all non-self S-RNase for ubiquitination and subsequent degradation, but it specifically leaves self S-RNase active to arrest the growth of self-pollen tube. A key question is whether SLF protein interacts with self- and non-self RNase differently, rendering only the former immune to degradation via Ub/26S proteasome. A modified inhibitor model proposed by Qiao *et al*. (2004a) supports this notion and is used to explain how AhSLF-$S_2$ recognizes self- or non-self S-RNase and leads to self-incompatibility or compatibility in the S-RNase-based SI reaction. After self S-RNase and non-self S-RNase are taken up into the growing pollen tube, AhSLF-$S_2$ interacts with both $S_1$- and $S_2$-RNases. The interaction of AhSLF-$S_2$ with self S-RNase, $S_2$-RNase, is somehow not effective in forming a functional SCF complex to ubiquitinate S-RNase, thus allowing its action in inhibiting the pollen tube growth. By contrast, the interaction of AhSLF-$S_2$ with non-self S-RNase, $S_1$-RNases, produces a functional SCF complex that in turn leads to its subsequent ubiquitination and destruction by the 26S proteasome. Recently, it was reported that hnRNP-U functions as a pseudosubstrate that binds to the corresponding F-box protein but is not destined for degradation by the SCF complex. (Davis 2002) Based on this example, it is possible that self S-RNase serves as a pseudosubstrate for $SCF^{SFB}$. The two variable regions located at the C terminus of SFB are implicated in binding with the pseudosubstrate self S-RNase.

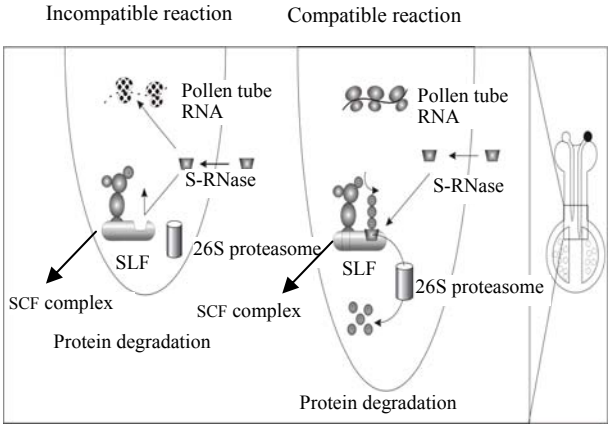


**Fig.3 Interaction model of pollination reaction at the molecule level**
**Note**: This pattern was developed from Seiji and Akira (2005).

Most of the known substrates of F-box proteins require phosphorylation for F-box recognition. (Vierstra 2003; Hershko and Ciechanover 1998) Together, Kunz *et al*. (1996) found that S-RNases could be phosphorylated by a calcium-dependent protein kinase from pollen tube without allelic specificity. Therefore, it needs to be tested what proteins affect the phosphorylation status of S-RNases and whether the phosphorylation condition will also affect the degradation of S-RNases.

Recently, Wang *et al*. (2005) detected that SLF protein is present in pollen cytoplasm during pollen tube growth in vitro via immunocytochemistry and Western blot techniques, thus they proposed that SLF protein is likely to play a key role in the elongating process of pollen tubes following pollination. Matsuura *et al*. (2001) and Ida *et al*. (2001) also reported the crystal structures of the $S_3$-RNase from the *Pyrus pyrifolia* and the $S_{F11}$-RNase from *N. alata*, respectively. Moreover, other additional proteins appear to be required for GSI response as well as male and female determinants, nevertheless the exact members of these proteins and how to function together with male and female determinants in GSI are not determined. Hence, structural analyses of *SLF/SFB*, further studies in S-RNases and correlative proteins would provide a deeper understanding of how the pollen S determinant functions in S-RNases-mediated GSI and self/non-self recognition mechanisms at the molecular level.

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